The Application of Paper Chromatography in Identifying Tuna Larvae

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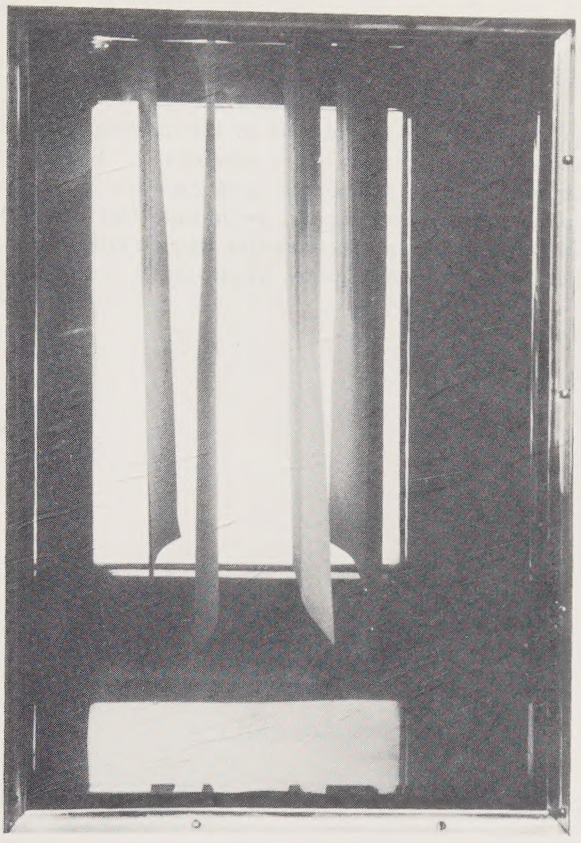

THE APPLICATION OF PAPER CHROMATOGRAPHY IN IDENTIFYING TUNA LARVAE

By

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ABSTRACT

In order to seek a quicker and easier means of identifying larvae of various species of tunas, experiments in paper partition chromatography were attempted. In this initial attempt the tests were limited only to determinations on the free amino acid content in the muscle tissue of these fishes. The results suggest that paper chromatographic technique has possible utility as a taxonomic tool for adult tunas. For the larvae, however, the results were rather inconsistent. It is believed that these inconsistent results were due to inadequate application of the technique rather than to failure of the technique itself.

CONTENTS

	Page
Introduction	1
Material	2
One-dimensional chromatograms	2
Procedure	2
Results	3
Two-dimensional chromatograms	4
Procedure	4
Results	4
Discussion and conclusions	6
Summary	8
Bibliography	9

ILLUSTRATIONS

FIGURE	Page
Frontispiece: Chromatograms being developed in chromatocab	
1. One-dimensional chromatograms of adult tuna samples developed in two different solvents	3
2. One-dimensional chromatograms of adult and larval samples of two species of tuna developed in n-butanol:methylethylketone:ammonia:water	4
3. Two-dimensional chromatogram of adult little tunny	4
4. Two-dimensional chromatogram of adult albacore	5
5. Two-dimensional chromatogram of adult bigeye	5
6. Two-dimensional chromatogram of adult yellowfin	6
7. Two-dimensional chromatogram of adult skipjack	6
8. Two-dimensional chromatogram of adult bigeye run in reverse order of solvents	7
9. Two-dimensional chromatogram of adult yellowfin run in reverse order of solvents	7
10. Two-dimensional chromatogram of larval yellowfin	8
11. Two-dimensional chromatogram of larval skipjack	8

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The species identification of larval and postlarval tunas has constituted an important segment of the research program of the Bureau of Commercial Fisheries, Honolulu Biological Laboratory. The usual method of identifying the larger juveniles and tracing certain diagnostic characters through a decreasing size series to the smallest available larva has been employed on some species with general success (Matsumoto, 1958; 1959). However, the identification of the younger larval stages becomes increasingly difficult as smaller and smaller larvae are examined. At the very earliest stages of development, it may be impossible to distinguish one species from a closely related one. To complicate matters further, there are a number of species of tunas such as bigeye (Parathunnus sibi), albacore (Thunnus germo), bluefin (Thunnus thynnus, and Thunnus maccoyi), dogtooth (Gymnosarda nuda), whose larval and postlarval forms have not been identified positively. In an attempt to find a faster and easier method of identifying some of these larvae, techniques employed in other fields of research were reviewed. One method which was considered worthy of investigation was that of paper chromatography.

Paper chromatography is an important technique used in the identification of chemical compounds. Its merit, apart from simplicity, is its extreme sensitivity, a small amount of a substance being easily detected under suitable conditions. Paper chromatography may be either one- or two-dimensional (for details, see Block, Le Strange and Zweig, 1952; Williams, 1954). In one-dimensional tests a sample of the solution to be analyzed is placed near one end of a strip of filter paper and that end of the paper is dipped into an appropriate solvent. Capillary action draws the solvent through the paper and through the spot where the sample solution had been applied. Each substance in the sample will move along with the solvent at a unique rate, such that all the substances in the sample will occupy a distinct position somewhere along the path of flow of the solvent. The rate of movement of each substance, and therefore the final position attained by each substance, in a given time, depends upon its solubility in the particular solvent used. Upon drying and treating the paper with a suitable indicator, the various substances appear as visible, colored spots. Substances within the sample which cannot be clearly separated or identified in one-dimensional chromatography may sometimes be separated by the two-dimensional method. In this a large sheet of paper, about 20 x 20 inches, is used. The paper is dried after the application of the first solvent and the side nearest the sample is then dipped into a second solvent. Upon completion of the development, the paper is dried and treated with a suitable indicator.

Initially the purpose of this study was (1) to explore the usefulness of paper chromatography in identifying the larvae and postlarvae of several species of closely related tunas, especially yellowfin (Neothunnus macropterus), bigeye and albacore, and then if possible, (2) to apply the method under field (shipboard) conditions.

On the assumption that the free amino acids in the muscle tissues of fishes are hereditary, the chromatograms obtained from samples of adults and larvae of the same species should show similarities. The plan of work followed, therefore, was first to determine the differences or similarities among the various species of adult tunas available for study; second, to compare the larval and adult samples; and third, to determine species differences among the tuna larvae.

Ordinarily in chromatographic work the concentration of specific substances in the samples tested would be determined by quantitative analysis. This was not considered worthwhile in this study, since, initially at least, I was interested only in determining if there were significant differences in the general chromatographic patterns of the amino acids found in tuna flesh. Consequently, possible variations due to locality of capture, between sexes, changes in amino acid complex with length of time the fish were kept in storage, and other variables were not considered.

The chemical analysis was done at the Hawaiian Sugar Planter's Association (HSPA) Experiment Station, where the paper chromatographic technique is regularly used. I wish to thank Dr. J. H. Payne, Principal Technologist at the HSPA Experiment Station, for the use of laboratory facilities and materials; Dr. Chen-Chuan Tu, Senior Technologist, for valuable technical advice; and chemists Richard M. Okamoto and George Akatsuka for aid in running the chromatograms. I am also grateful to Tamotsu Nakata, Scientific Illustrator at the Honolulu Biological Laboratory, for preparing the figures.

MATERIAL

Samples of fresh muscle tissue of adult tunas captured in Hawaiian waters were obtained at the Honolulu fish market and from the Bureau of Commercial Fisheries research vessel Charles H. Gilbert. It was not possible to collect uniformly fresh samples, since those obtained at the market had been kept chilled from 1 to 10 days, whereas those taken on the research vessel were frozen immediately after the completion of fishing operations. Samples from the fish market included yellowfin, bigeye and albacore, and those from the vessel consisted of skipjack (Katsuwonus pelamis) and little tunny (Euthynnus yaito). Two species of frigate mackerels (Auxis thazard and A. thynnoides), which were taken on November 30, 1957 from the Bureau's research vessel, John R. Manning, and kept frozen for about 8 months, were also used in this study. Fresh samples of these two species were not immediately available.

Samples of tuna larvae were obtained from zooplankton collections taken by the Gilbert working in Hawaiian waters, and from another of the Bureau's research vessels, Hugh M. Smith working in Hawaiian and equatorial waters. The zooplankton samples were frozen aboard the vessels immediately upon capture. At the laboratory, the samples were thawed and tuna larvae and postlarvae were sorted out in a bath of ice water under a dissecting microscope. The larvae were refrozen for later use. Because they could be readily identified visually, only yellowfin and skipjack larvae were employed in this preliminary work. These larvae measured 4.5 to 10.2 mm. in total length.

Solvents used in the experiments were some of those which had been used successfully by other workers in separating amino acids. They were (1) 80 percent solution of phenol in water, (2) m-cresol: acetic acid:water (48:2:50), (3) n-butanol:methylethylketone: 17 N ammonia: water (5:3:1:1) and (4) n-butanol:acetic acid:water (4:1:5). A 0.1 percent solution of ninhydrin in 95 percent ethanol was used as the indicator solution to visually detect the amino acid spots on the paper.

Whatman No. 1 filter paper was used throughout the experiments. For one-dimensional chromatograms, 7 x 22-inch sheets of paper were used. For two-dimensional work, 18 1/2 x 22-inch sheets were used.

ONE-DIMENSIONAL CHROMATOGRAMS

Procedure

In one-dimensional chromatography the samples were placed about 1 inch apart in a row near one end of the filter paper. They were allowed to dry at room temperature and the amino acids were separated in a descending chromatogram by dipping the end of the paper bearing the samples into a suitable solvent. In order to intensify the color of the various amino acids present, the paper was heated in a convection oven at about 80°C. for 10 to 15 minutes after applying the indicator solution. The outline of each acid spot was then marked with pencil.

The first series of chromatograms was intended to define sample size and solvent appropriate to the clear separation of amino acids present in adult muscle tissue. Phenol was used as the solvent for the initial tests which consisted of 10 samples. Two of these samples consisted of small pieces of muscle tissue (approximately 8 mm.^{3}) pressed directly onto the filter paper. Although excess tissue was removed, considerable streaking occurred and there was no separation of the acids. Samples of fluid which were squeezed from muscle tissue varying in size from 1 to 30 µl. were also tested. Of these, only the 1 µl. sample appeared promising. Therefore, further tests were made on this sample size with a sample from each of 6 species of tunas. The chromatograms showed some streaking, and the separation of the amino acids was indistinct.

A second solvent, m-cresol:acetic acid: water, was tried with a 1 µl. sample from each of the previous 6 species. Reasonably good separations were obtained, but the spots were not well defined and were of the type known as "comets."

A third solvent, a solution of n-butanol: methylethylketone: 17 N ammonia:water, also was tested as before. With this solvent, excellent separation was obtained from samples of

all of the species. Larvae of two species of tunas, skipjack and yellowfin, measuring from 5.5 mm. to 10.5 mm. were used for these tests. Four skipjack samples consisting of 1, 2, 3 and 5 larvae, and three yellowfin samples consisting of 1, 2 and 3 larvae were tested. In addition, samples of muscle tissue fluid from adults of skipjack and yellowfin were used as controls. Each larval sample was mashed on the filter paper with the end of a glass rod, and the tissue was removed to prevent streaking around the point of application.

In addition to the three solvents mentioned, n-butanol:acetic acid:water was also tried and excellent separation was obtained with them. Of the four solvents used, the two n-butanol solutions provided the best results, m-cresol was fair, and phenol was not satisfactory.

Another series of chromatograms was prepared in order to determine the proper size of the larval sample required for clear separations. In this series n-butanol:methylethylketone: 17 N ammonia:water, which had proved successful for the adult samples, was used as the solvent. There was good separation in the 2- and 3-larvae samples, but only faint traces were obtained from samples with 1 and 5 larvae. Thus, it was decided that a sample size of about 3 larvae was adequate.

Results

Interesting results were obtained in the chromatograms developed in the two n-butanol solvents (fig. 1). In solvent A (n-butanol:acetic acid:water) it was possible to distinguish the adults of the two frigate mackerels from the other tunas by the smaller number (5) and position of the spots. However, the two species did not differ much from each other. It is not possible to state how much of this difference was due to the long period (about 1 year) the frigate mackerels had been kept frozen. Of the other species, albacore showed 7 spots and was easily separated from bigeye, yellowfin and skipjack, which had nearly identical patterns of 8 spots each. All of these samples, except skipjack were obtained from the fish market.

Somewhat different results were obtained when the same samples were run in solvent B (n-butanol:methylethylketone:ammonia:water). The two frigate mackerels had more spots (8) than in solvent A. The positions of the spots differed, indicating the presence of additional amino acids not shown by solvent A. Of the other species, bigeye and albacore had fewer spots (6 and 7, respectively) and their positions differed, but there was no change in the number of spots in yellowfin and skipjack.

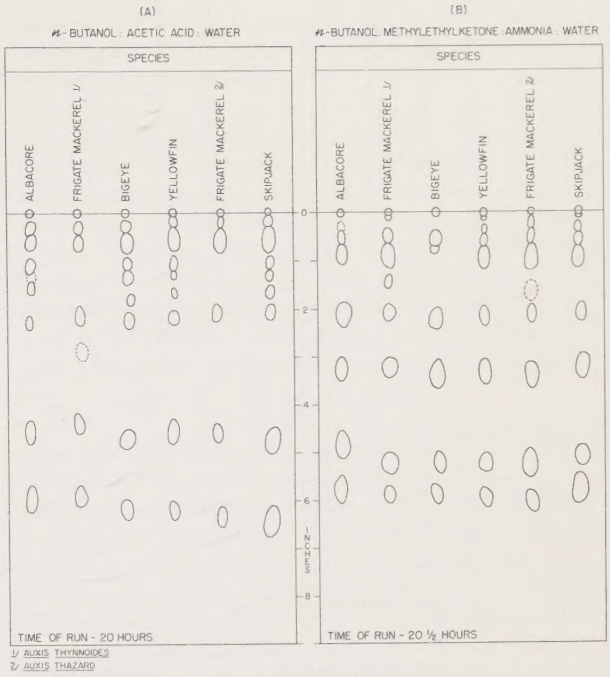

Figure 1.--One-dimensional chromatograms of adult tuna samples developed in two different solvents.

Considering the number and location of amino acid spots revealed with the two solvents, it is possible to differentiate the adult albacore and bigeye from the others. The two frigate mackerels can be distinguished from the other species by the smaller number of spots in solvent A and from each other by the position of the spots in solvent B. The yellowfin and skipjack can be distinguished from other species, but not from each other.

Chromatograms of the larvae of yellowfin and skipjack were generally comparable to that of the adults, but exhibited wide variations in the number of spots obtained. Five out of 9 sample applications (1- and 3-larvae samples) produced good separation, showing 7 or 8 spots, 2 sample applications (1-larva samples) produced no spots and 2 sample applications (1- and 5-larvae samples) produced 3 or 4 spots. In all instances where a sufficient number of spots (8) were obtained, the positions of the spots generally coincided with those of the respective adult traces (fig. 2). However, inasmuch as the adults of these two species yielded similar results, it was not surprising to find no significant difference in the larval chromatograms of the two species.

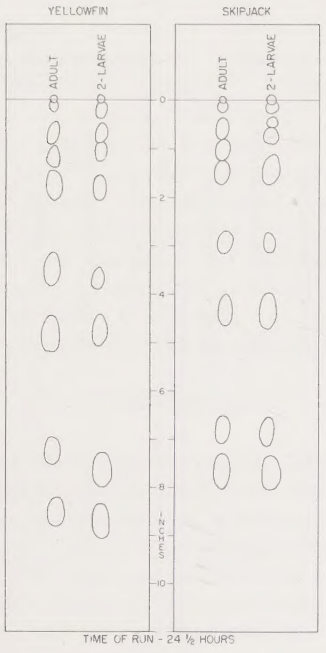

Figure 2. --One-dimensional chromatograms of adult and larval samples of two species of tunas developed in n-butanol:methylethylketone:ammonia:water.

TWO-DIMENSIONAL CHROMATOGRAMS

Procedure

In two-dimensional chromatograms the sample to be analyzed was placed in one corner of a large (18 x 22 inches) sheet of filter paper. The end of the paper nearest the sample was placed in the first solvent until the solvent front had descended to the opposite edge. After drying, an edge of the paper adjacent to the sample was dipped into a second solvent and allowed to develop as in the first instance. The paper was then dried and treated with the indicator solution.

Because successful separation of the amino acids was obtained with n-butanol:methylethylketone: 17 N ammonia:water and n-butanol: acetic acid:water in one-dimensional chromatography, these solvents were used in the order mentioned. The samples of adults were prepared by squeezing out the muscle fluid by hand. Larval samples consisted of whole tuna larvae which were squeezed directly on the paper with a glass rod. Samples from adults of 5 species of tunas (yellowfin, bigeye, albacore, skipjack and little tunny) and the larvae of 2 species of tunas (yellowfin and skipjack) were developed for 21 to 24 hours in each solvent. The amount of sample used was 10 µl. of muscle tissue fluid from each of the adults and 2 to 7 larvae of each of the latter two species.

Results

The results of the two-dimensional chromatograms were not always satisfactory and varied considerably from one experiment to another. In many instances the variations were extreme, from 0 to 11 or 12 spots, even among samples taken from fish of the same species. Part of these variations were probably due to the inexact manner in which the samples had been prepared. The comparisons discussed here are based only on those chromatograms which yielded the maximum spots per species, and may be somewhat arbitrary. Nevertheless, they give us some idea whether this method is applicable in species identification of the tunas.

Figures 3-7 show five of the better traces obtained with samples from adult tunas. Some differences were evident between little tunny, albacore, bigeye and skipjack, but differences between bigeye and yellowfin could not be determined, since the trace of the latter species showed some streaking and the separation of the spots was not sufficiently clear.

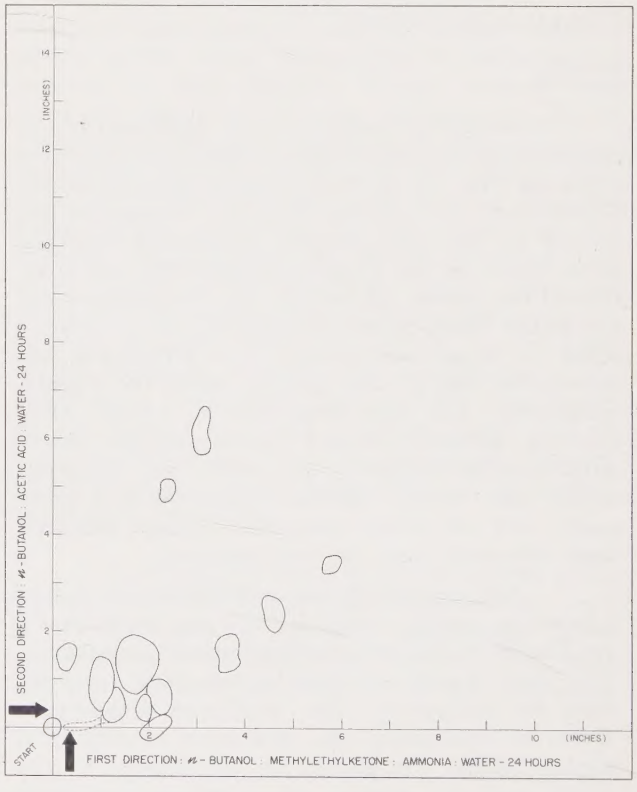

Figure 3. --Two-dimensional chromatogram of adult little tunny.

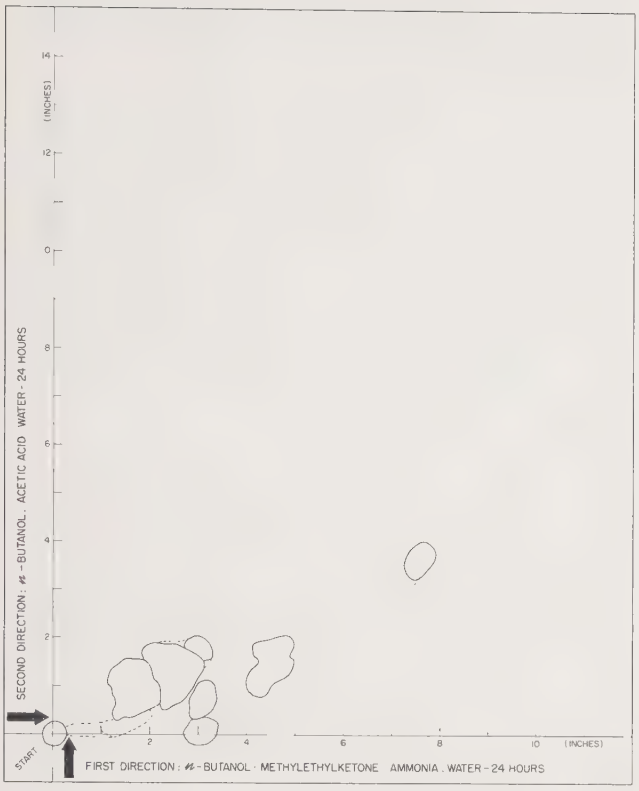
SECOND DIRECTION: n-BUTANOL . ACETIC ACID WATER - 24 HOURS
(INCHES)
START
FIRST DIRECTION: n-BUTANOL . METHYLETHYLKETONE AMMONIA . WATER - 24 HOURS

Figure 4.--Two-dimensional chromatogram of adult albacore.

A second testing of the bigeye and yellowfin samples produced better separation of the spots (figs. 8 and 9). In this instance, the order of solvents was inadvertantly reversed, but it is not believed that this had any adverse effect on the chromatograms. Both species showed slightly different patterns than those run in the original order of solvents. However, the differences, if any, between the two species were insignificant.

Some discrepancy is noted in the number of spots obtained with identical solvents between the one- and two-dimensional chromatograms, i.e., the two-dimensional chromatograms contained less spots in each of the solvents than the one-dimensional chromatograms run in identical solvents. This discrepancy could perhaps be due to difference in the time of runs (1 month apart) between the two tests. There is also the possibility that some of the amino acids separated in one solvent may have been lost in the other solvent in the two-dimensional tests. Because the amino acids were not identified, it is not possible to say whether the acids lost in each of the two dimensions were of the same type for all of the samples. However, inasmuch as this would involve a specific action between solvent and acid, it is logical to assume that identical acids were lost by all of the species. If so, this discrepancy should not affect the final results.

Both good and poor results were obtained from the samples of larval tunas. In the case of yellowfin (fig. 10), no trace was obtained with samples of less than 5 larvae and even with this number, the sample size seemed to be too small. Perhaps the use of more larvae would have given better results, but the supply of larval yellowfin was depleted at this stage of the investigation and further tests were not possible.

In the case of skipjack (fig. 11), however, better results were obtained. The figure shows the chromatogram of a sample of 7 larvae, their sizes ranging from 4.5 to 8.0 mm. Although not all of the amino acids shown by the adults were obtained from the larvae, a sufficient number was developed to show some similarity in the overall patterns of the dominant amino acids.

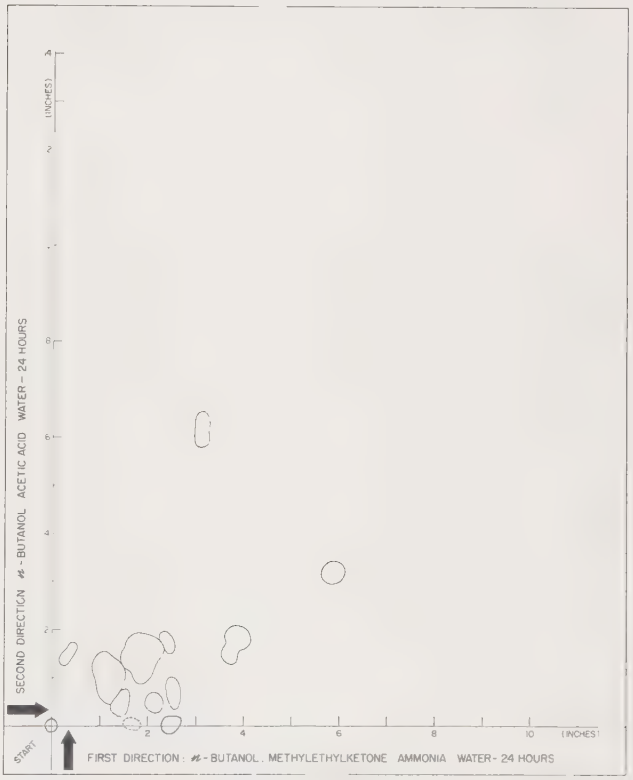
SECOND DIRECTION: n-BUTANOL . ACETIC ACID WATER - 24 HOURS
(INCHES)
START
FIRST DIRECTION: n-BUTANOL . METHYLETHYLKETONE AMMONIA WATER - 24 HOURS

Figure 5.--Two-dimensional chromatogram of adult bigeye.

SECOND DIRECTION : n - BUTANOL : ACETIC ACID WATER - 24 HOURS
(INCHES)
START
FIRST DIRECTION : n - BUTANOL METHYLETHYLKETONE AMMONIA WATER - 24 HOURS

Figure 6. --Two-dimensional chromatogram of adult yellowfin.

DISCUSSION AND CONCLUSIONS

These experiments provided valuable information concerning the use of chromatographic technique in the species identification of some of the tunas. It was found that adult samples of the same species of tuna developed one-dimensionally in different solvents resulted in dissimilar chromatograms. This was particularly noticeable of the albacore and bigeye samples. Although yellowfin and skipjack samples developed in two different solvents showed no difference in the chromatograms, it is believed that trials with other solvents would reveal a suitable solvent capable of producing different chromatograms between these two species. Similarly, suitable solvents also could be sought for the two frigate mackerels and other species of tunas not mentioned. If successful, one-dimensional chromatography might be a useful taxonomic tool for adult tunas.

The same cannot be said with equal assurance about larval identification because larvae of only two species (yellowfin and skipjack) were tested. However, one encouraging observation, the general similarity of adult and larval chromatograms, was noted in the results of the larval tests.

As expected, adult samples developed successfully in two-dimensions yielded more spots than those developed in only one-dimension, and differences in the position of the spots were more pronounced among most of the species. Only two out of the six species tested could not be distinguished, since chromatograms of yellowfin and bigeye samples appeared identical. Perhaps the use of other solvents may provide visible differences between these two species.

Results of larval samples of yellowfin and skipjack were rather inconsistent. However, they do not preclude the usefulness of this technique in taxonomic studies. Part of the inconsistency could have been due to inadequate methods of application which can be remedied. There is some encouragement in the fact that, of the better results obtained with skipjack larvae, some similarity in the general distributional pattern of the more prominent amino acids were evident.

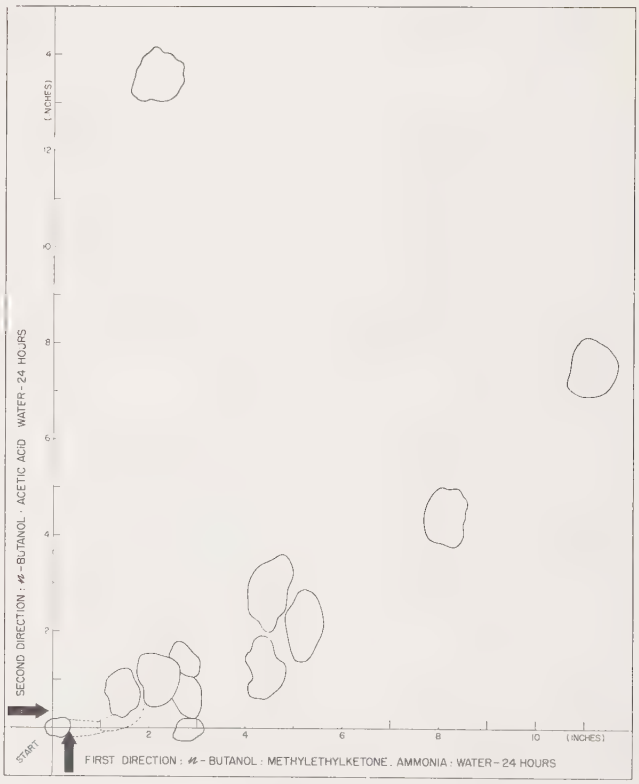
SECOND DIRECTION : n - BUTANOL : ACETIC ACID WATER - 24 HOURS
(INCHES)
START
FIRST DIRECTION : n - BUTANOL : METHYLETHYLKETONE . AMMONIA : WATER - 24 HOURS

Figure 7. --Two-dimensional chromatogram of adult skipjack.

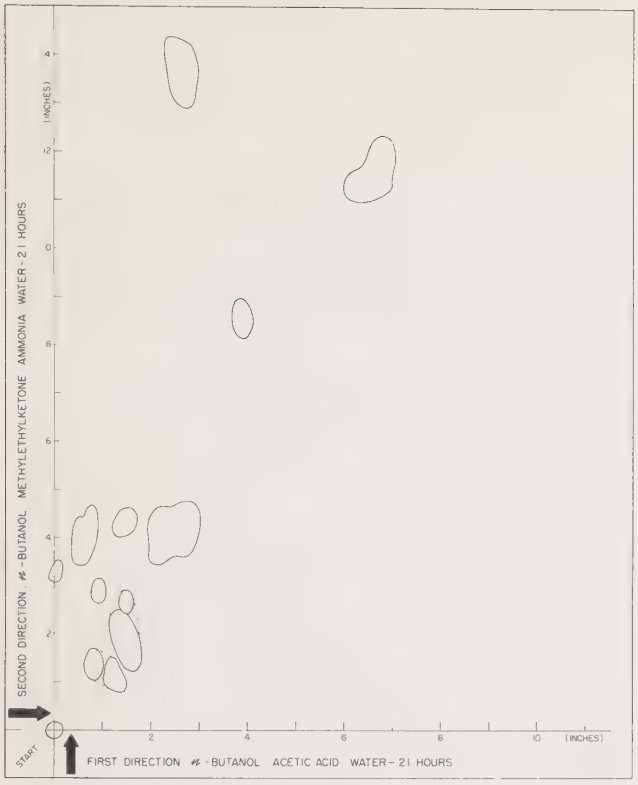
SECOND DIRECTION - #-BUTANOL METHYLETHYLKETONE AMMONIA WATER - 21 HOURS
(INCHES)
START
FIRST DIRECTION - #-BUTANOL ACETIC ACID WATER - 21 HOURS
(INCHES)

Figure 8.--Two-dimensional chromatogram of adult bigeye run in reverse order of solvents.

The only definite and consistent difference between adult and larval samples in both one- and two-dimensional chromatograms was the presence of a dark greenish-brown spot in all the adult samples. This contrasted with the purplish color exhibited by all other amino acids. The spot, which was identified as aspartic acid from chromatograms of 14 known amino acids developed in identical solvents as the test samples, was not found in any of the larval samples. Inasmuch as adult samples of all the species tested showed this particular amino acid, it is believed that this acid reflects growth rather than species difference.

There are several hypothetical reasons which may account for the inconsistency in the results of the larval samples. One of these is the probable reduction of amino acid concentration in the larvae owing to leaching. Larvae used as samples had been frozen and thawed repeatedly from the time they were first captured to the time they were tested. Inasmuch as the free amino acids found in animal tissues are water soluble, it is reasonable to believe that much of the amino acids had been lost during the thawing processes.

Another reason is the possibility that both leaching and partial decomposition of the amino acids still remaining in the tissue may have occurred in larvae which had been dead prior to capture. That such incidence of mortality among the larvae prior to capture is occasionally quite extensive in restricted localities was shown by Strasburg (1959), who found as much as 99 percent mortality in the larvae of frigate mackerel (Auxis sp.) at one locality.

There is also a remote possibility that part of the inconsistency could have been the result of variations in the amino acid complex caused by changes in the diet of the larvae. Evidence of such variations in fish was found by Farris (1958) in experiments with controlled feeding of a sardine (Sardinops caerulea). Whether dietary changes of such magnitude as to cause variations in the amino acid complex of tuna larvae do occur in nature, or the effect this change has on the survival of the larvae is not yet known. However, inasmuch as the possibility does exist, such factors as this must be considered.

In view of the limitations in the experimental design and the failure in obtaining equally fresh adult samples of all the species tested,

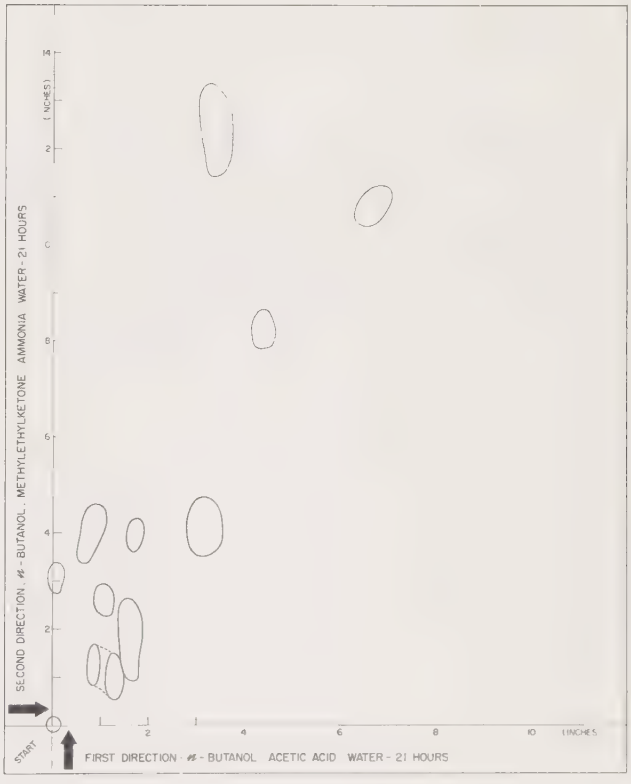
SECOND DIRECTION - #-BUTANOL METHYLETHYLKETONE AMMONIA WATER - 21 HOURS
(INCHES)
START
FIRST DIRECTION - #-BUTANOL ACETIC ACID WATER - 21 HOURS
(INCHES)

Figure 9.--Two-dimensional chromatogram of adult yellowfin run in reverse order of solvents.

Figure 10.--Two-dimensional chromatogram of larval yellowfin.

the author feels that any conclusion on the use of the technique in species identification of tunas would be premature. The technique is not without promise, however, as was noticed by the difference in positions of spots in the two frigate mackerels, which had been captured at the same time and kept frozen an equal length of time. Also, the close resemblance between adult and larval skipjack, both of which had been frozen immediately upon capture, is significant.

Comparing the number of spots shown by adult species other than skipjack with those developed with fresh-frozen skipjack, it is believed that either the changes due to proteolytic enzyme reaction may not have been as important as suspected, or that the suspicion that these fish had been taken at such widely differing time as to cause noticeable changes in the amino acid complex could have been unfounded. If so, the data obtained are certainly encouraging.

SUMMARY

1. Both one- and two-dimensional chromotography on paper were attempted: first, to see if the various species of adult tunas possessed characteristic compositions of free amino acids; second, to determine if the known tuna larvae possessed the same free amino acids as their respective adults; and third, to develop, if possible, a simplified shipboard technique.

2. Instead of trying to identify each specific amino acid found in the muscle tissue of these fish, emphasis was placed upon seeking characteristic differences in the overall chromatographic patterns of the amino acids among the various species of tunas tested.

3. Samples consisted of small bits of muscle tissue (approximately 8 mm.^{3}) and minute quantities of fluid squeezed from muscle tissues. They were placed directly upon the filter paper.

4. Four different solvents were tested, with n-butanol:acetic acid:water in a ration of 4:1:5 and n-butanol:methylethylketone: 17 N ammonia:water in a ratio of 5:3:1:1 showing the best results in both one- and two-dimensional chromatograms. All experiments were made on Whatman No. 1 filter paper.

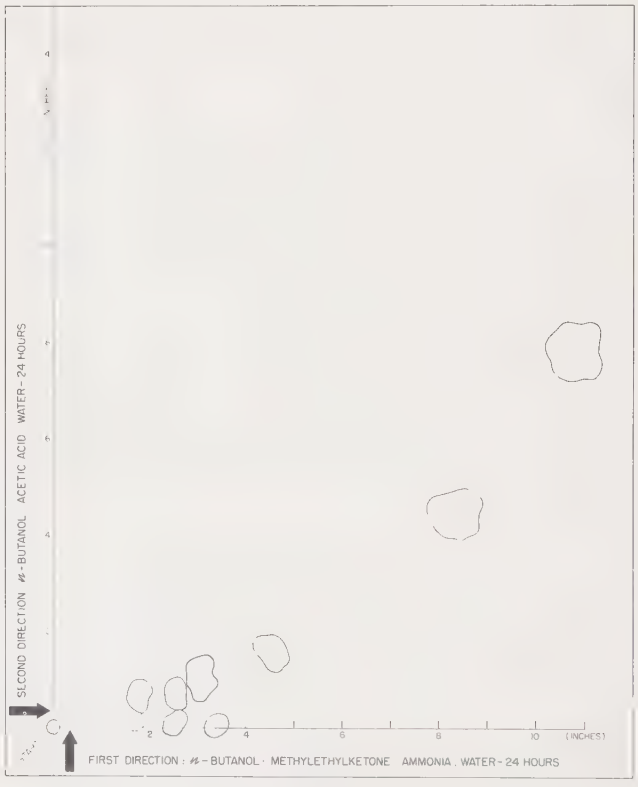

Figure 11.--Two-dimensional chromatogram of larval skipjack.

5. Giving due consideration to the fact that the time of capture was unknown for all of the samples and that the treatment of the samples had not been uniform, it is suspected that this technique may have useful possibilities in taxonomic studies of the adults.

6. Chromatograms of the larvae, in repeated runs of samples of the same species, showed varying results. The inconsistencies in these results may, in part, have resulted from the loss of free amino acids from the larvae due to leaching after death, prior to their capture, or if taken alive, to the repeated freezing and thawing they were subjected to while they were being segregated from the rest of the plankton. Although the amount and effect of decomposition of the amino acids in the larval samples were not investigated, this possibility cannot be ignored.

7. The author feels that any conclusion regarding the use of chromatographic technique, based on the present experiments, is premature. Nevertheless, he believes that methods of applying this technique to taxonomic studies of adult and larval tunas can be developed.

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